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Glutamine Synthetases of Higher Plants[1]

EVIDENCE FOR A SPECIFIC ISOFORM CONTENT RELATED TO THEIR POSSIBLE PHYSIOLOGICAL ROLE AND THEIR COMPARTMENTATION WITHIN THE LEAF

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SHEILA F. MCNALLY, BERTRAND HIREL, PIERRE GADAL, A. FRED MANN, AND GEORGE R. STEWART
School of Botany, Trinity College, University of Dublin, Dublin 2, Eire (S. F. McN.); Physiologie Végétale Métabolique, ERA au CNRS No. 799, Université de Paris Sud, Bat 430, 91405 Orsay Cedex, France (B. H., P. G.); and Department of Botany, Birbeck College, University of London, London WC1E 7HX, Great Britain (A. F. M., G. R. S.)

ABSTRACT

The chromatographic properties of glutamine synthetase isoforms have been investigated in a wide range of higher plant leaves and shoots using ion exchange chromatography. Different patterns of glutamine synthetase isoform content were observed. Among higher plants, four patterns or groups could be recognized. The first group is characterized by having only cytosolic glutamine synthetase, whereas the second group is distinguished by having only chloroplastic glutamine synthetase. The third group is characterized by cytosolic glutamine synthetase being a minor component of the total leaf glutamine synthetase activity. The fourth group is distinct from the other groups in having high cytosolic and chloroplast glutamine synthetase activity. Immunological studies have been undertaken on a few species from each group to identify unambiguously both cytosolic and chloroplastic glutamine synthetases.

It is now established that glutamine synthesis may occur in two compartments of the leaf cells of most higher plants (1, 6–8, 15, 16, 27). Chromatographic and electrophoretic techniques combined with subcellular localization studies have demonstrated the presence of two different isoforms of GS[2] in green tissue (1, 6–8, 13, 15, 16, 18, 24). One isoform, designated GS_1, is localized in the cytosol, whereas the other, designated GS_2, is found in the chloroplast (7–9, 15). The isoforms have been characterized by their specific kinetic and regulatory properties. They exhibit differences in their pH optima, heat stability, and K_m for glutamate (6, 13, 15).

Within the small number of plant species studied such as barley (15), rice (6), pea (7), sorghum (8), spinach (9), and pumpkin (13), differences have been observed in GS isoform complement. In spinach (9), only GS_2 is present, whereas in other C_3 plants like rice (6), pea (7), and barley (15), a minor GS_1 component is present (up to 20% of total enzyme activity). In sorghum, a C_4 plant, the pattern is yet again different with GS_1 representing about 70% of total GS activity in the leaf (8). We have extended these studies to a wider range of higher plants including halophytes and parasitic plants to confirm differences in the relative proportions of GS_1 to GS_2.

Immunological methods were also employed with selected plant species to identify very specifically the cytosolic and chloroplastic form of GS.

MATERIALS AND METHODS

Plant Culture. Most plants were grown in plastic containers filled with vermiculite and watered daily for 3 weeks before harvesting, except for *Alnus glutinosa* which was grown for 6 months, with a complete Hoagland solution (11) in a controlled environment chamber (7). Tropical grasses were grown on a Lockard solution (14) under controlled conditions (6). CAM plants were grown for 8 weeks at the Phytotron of Gif-sur-Yvette (France) on a nutrient solution (4). Parasitic plant species were collected or grown as previously described (18). Halophytic plant species were collected from a salt marsh at Ichenore (West Sussex, England) or grown from seed in John Innes Compost No. 3 (1).

Enzyme Extraction and Purification. All operations were carried out at 4°C. Ten to 20 g of fresh leaves (groups B, C, and D) or shoots (group A) were used in each experiment. For most plants, the grinding medium (100 ml) contained 25 mM Tris-HCl buffer (pH 7.6), 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM DTT, and 1 g Polyclar (acid-washed). Leaves or shoots were homogenized with a Polytron PT10 mixer at maximum speed for 4 × 15 s. The brei was filtered through two layers of gauze and then centrifuged at 45,000*g* for 30 min. The supernatant was directly layered on the top of a DEAE-Sephacel column (Pharmacia, Uppsala) (1 × 10 cm) previously equilibrated in the homogenizing buffer without Polyclar. A linear gradient of 0 to 400 mM NaCl or 0 to 600 mM KCl dissolved in 200 ml of the equilibrating buffer was used to elute the proteins. Four-ml fractions were collected and the flow rate adjusted to 20 ml h^{-1}.

The purification procedure for the following plants was modified as follows.

Alnus glutinosa. The grinding medium was 50 mM Tris-HCl,

Table I. *Plants Exhibiting Only GS_1 Activity: Group A*

Plant Species
Cuscuta australis (P)[a]
Lathraea clandestina (P)
Lathraea squamosa (P)
Orobanche cernua (P)
Orobanche hederae (P)
Orobanche minor (P)
Orobanche ramosa (P)

[a] P, parasite.

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[2] Abbreviations: GS, glutamine synthetase; GS_1, cytosolic glutamine synthetase; GS_2, chloroplastic glutamine synthetase.

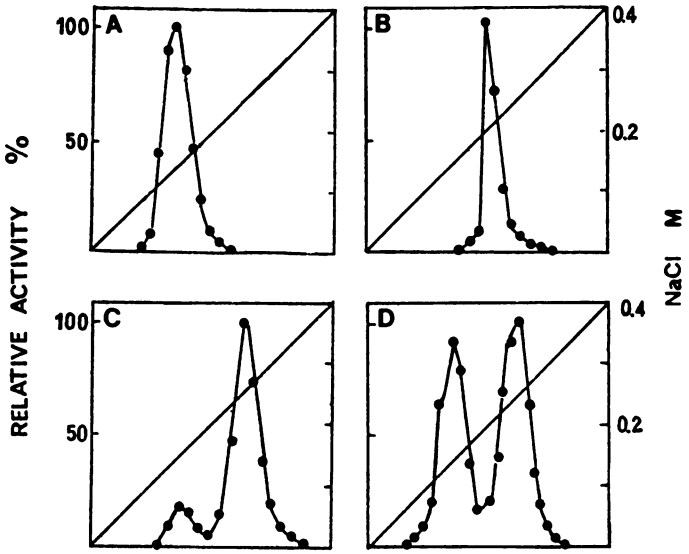

FIG. 1. Representative elution profiles of GS_1 and GS_2 of the four major groups of plants after chromatography on DEAE-Sephacel ion-exchange resin. Results are expressed as a percentage of total activity in the leaf. Recovery of GS activity after chromatography was always greater than 80% of the total activity initially loaded into the column. A, Group A. The achlorophyllous parasite *Cuscuta australis* (Table I). Maximum activity is 0.13 nkatal g^{-1} fresh wt. B, Group B. The solanacae *Lycopersicon esculentum* (Table II). Maximum activity is 0.66 nkatal g^{-1} fresh wt. C, Group C. The legume *Phaseolus vulgaris* (Table III). Maximum activity is 0.45 nkatal g^{-1} fresh wt. D, Group D. A tropical grass *Chloris gayana* (Table IV). Maximum activity is 0.58 nkatal g^{-1} fresh wt.

Table II. *Plants Exhibiting Only GS_2 Activity: Group B*

Plant Species
Ammophila arenaria (G)[a]
Atriplex hastata (H)
Atriplex lanceniata (H)
Atriplex patula (H)
Cakile maritima (H)
Crithmum maritimum (H)
Croixlacrima jobi
Croton megacarpum
Drepenocarpus lunatus
Eriophorum angustifolium (G)
Frankenia laevis (H)
Halimione portulacoides (H)
Lemna minor
Limonium vulgare (H)
Lupinus sp. (L)
Lycopersicon esculentum
Nicotiana tabacum
Petunia grandiflora
Salicornia europea (H)
Senecio vulgaris
Spinacia oleracea
Suaeda maritima (H)
Marchantia polymorpha (LP)
Osmunda regalis (LP)

[a] G, Graminae; H, halophyte; L, legume; LP, lower plant.

(pH 7.8), containing 2 mM $MgCl_2$, 2 mM EDTA, 50 mM 2-mercaptoethanol, 1 mM DTT, 2% (w/v) soluble PVP (PVP40, Sigma), and 2 g Polyclar (acid-washed). After filtration and centrifugation, the brei was diluted twice with cold distilled H_2O and directly applied onto a DEAE-Sephacel column.

Kalanchoë blossfeldiana and Kalanchoë daigremontiana. The

Table III. *Plants Exhibiting GS_1 Activity Equal to or Lower than 30% of Total Activity: Group C*

Relative proportions of GS_1 and GS_2 are expressed as a percentage of total activity in the leaf.

Plant Species	GS_1	GS_2
	%	
Alnus glutinosa (T)[a]	30	70
Aster tripolium (H)	27	63
Avena sativa (G)	10	90
Dendrobium nobile (O)	22	78
Festuca rubra (H)	10	90
Hordeum vulgare (G)	17	83
Oryza sativa (G)	18	82
Panicum maximum (C_4 PCK)	15	85
Phaseolus aureus (L)	25	75
Phaseolus vulgaris (L)	5	95
Pisum sativum (L)	15	85
Plantago maritima (H)	9	91
Puccinelia maritima (H)	20	80
Spergularia marina (H)	15	85
Triticum aestivum (G)	18	82

[a] T, tree; H, halophyte; G, Graminae; O, orchid; C_4 PCK, plant with a C_4 carbon metabolism pyruvate carboxykinase type; L, legume.

Table IV. *Plants Exhibiting GS_2 Activity Greater than 30% of Total Activity: Group D*

Relative proportions of GS_1 and GS_2 are expressed as a percentage of total activity in the leaf.

Plant Species	GS_1	GS_2
	%	
Chloris gayana (C_4 PCK)	45	55
Digitaria sanguinalis (C_4)	80	20
Echinochloa crus-galli (C_4)	71	29
Erythrina abysinica (TL)	70	30
Erythrina cristagalli (TL)	51	49
Erythrina corallodendon (TL)	45	55
Erythrina flabelliforme (TL)	68	32
Kalanchoë blossfeldiana (CAM)	65	35
Kalanchoë daigremontiana (CAM)	36	64
Pennisetum americanum (C_4)	50	50
Sorghum vulgare (C_4)	70	30
Zea mays (C_4)	45	55

[a] C_4 PCK, plant with a C_4 carbon metabolism pyruvate carboxykinase type; C_4, plant with a C_4 carbon metabolism; TL, tropical legume.

grinding medium was 200 mM Tris-HCl (pH 8.0) containing 1 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM DTT, 2% (w/v) PEG 20,000 (Serva). After filtration and centrifugation, the brei was dialyzed against a large excess of 10 mM Tris-HCl (pH 7.6) containing 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM DTT for 12 h and then applied to a DEAE-Sephacel column equilibrated in the same buffer.

Halophytic Species. Crude extracts were first desalted on G25 equilibrated with extraction buffer (minus Polyclar) before chromatography on DEAE-Sephacel).

Enzyme Assays. Determination of transferase and synthetase activities of GS were as described by Rhodes *et al.* (23).

Immunodiffusion and Immunoprecipitation. Immunodiffusion and immunoprecipitation experiments were performed as previously described (9).

RESULTS AND DISCUSSION

Four groups of plants can be distinguished by their specific GS isoform content. The first group of plants (Table I, group A) is

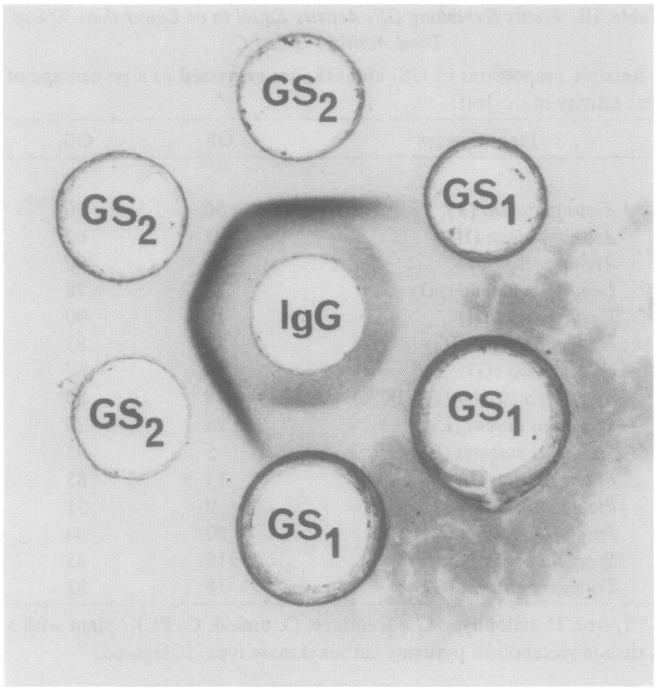

FIG. 2. Immunochemical characterization of GS_1 and GS_2 from pea leaves. Immunodiffusion was performed on agar plates (2%) according to the Ouchterlony technique (21). Three wells on the left contained 10 μl of concentrated GS_2 and three wells on the right contained 10 μl of concentrated GS_1. The central well contained 10 μl of antiserum raised against GS from spinach chloroplasts (IgG). The plates were placed for 48 h at 4°C in a humid atmosphere and rinsed for 29 h in 0.9% NaCl solution. The plates were dried under filter paper at room temperature, stained for 1 h with Coomassie Brilliant Blue R, and destained overnight. Similar results were also obtained with GS_1 and GS_2 isolated from rice, sorghum, and *Kalanchoë* leaves.

distinct in having only one peak of GS activity eluting at about 0.15 M NaCl concentration (Fig. 1A). Similar elution values have been obtained for GS_1 from various plants (1, 6–8, 15, 16). This suggests that only GS_1 is present in this group of achlorophyllous higher plant parasites which are nutritionally dependent upon their hosts (19). The second group (Table II, group B) is characterized by the apparent absence of GS_1, a pattern already described for spinach (9). It can be seen in Figure 1B that a single peak of enzyme activity is present but which is eluted at a higher ionic strength of about 0.22 M NaCl. This elution profile is typical for example, of a number of chenopods from various environments, including salt marsh habitats. It is interesting to note that two lower plants, *Marchantia* and *Osmunda*, also possess only GS_2. The third group (Table III, group C) is distinguished in having two GS isoforms, with a minor GS_1 (5–30% of total leaf enzyme activity) eluting at about 0.15 M NaCl and a major GS_2 (70–95% of total leaf enzyme activity) which is eluted at about 0.25 M NaCl (Fig. 1C). This pattern of GS activity is typical of many C_3 grasses and some temperate legumes. It is noteworthy that some halophytic plant species possess both GS isoforms, suggesting that GS isoform complement is not necessarily dependent upon environmental conditions. In the fourth group (Table IV, group D), GS_1 and GS_2 are also present, eluting at 0.15 and 0.25 M NaCl concentrations, respectively (Fig. 1D), but as shown in Table IV, the contribution of GS_1 is consistently higher than that found in Group C (Table III). In corn, for example, GS_1 activity represents about 45% of total enzyme activity in the leaf, and in *Digitaria* the contribution of GS_1 is much higher, up to 80% of total GS. This pattern is observed for most C_4 plants, a CAM plant, *Kalanchoë*, and some tropical legumes such as *Erythrina*.

Immunodiffusion and immunoprecipitation were employed to characterize unambiguously GS_1 and GS_2 isoforms from some of the plant species examined using antibodies raised against chloroplastic GS from spinach leaves (9). It can be seen in Figure 2 that these antibodies recognize the chloroplastic GS from pea leaves but also GS_1, the cytosolic enzyme, with a faint band of precipitate. Furthermore, it can be observed that a partial identity exists between the two proteins with a spur occurring between the two precipitin bands. Similar results were also obtained, *e.g.* in rice, sorghum, and *Kalanchoë* which differ in their relative proportions of GS isoform content. The immunological behavior of GS_1 and GS_2 was also studied by immunoprecipitation. GS_1 and GS_2 were separated by ion-exchange chromatography on DEAE-Sephacel and immunoprecipitated. Constant amounts of enzyme are incubated with increasing concentrations of antibodies and the enzyme activity is measured in the supernatant following the removal of the immune complexes. Figure 3B shows the results obtained with rice; similar results were observed with GS_1 and GS_2 isolated from pea and sorghum leaves. For GS_1, only 40% of total enzyme activity is precipitated with 200 μl of antibodies. GS_2, on the other hand, is completely precipitated with 100 μl of

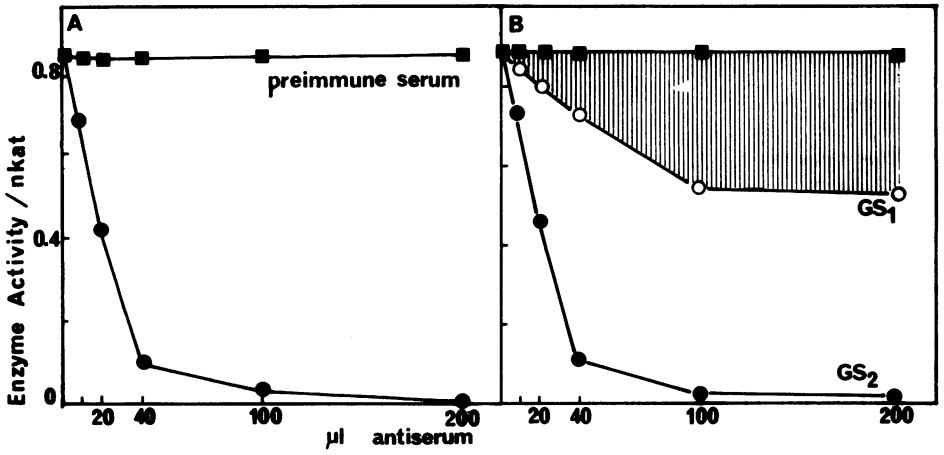

FIG. 3. Immunoprecipitation curves of GS from spinach (A) and rice (B) leaves. In B, GS_1 and GS_2 were separated by using DEAE-Sephacel chromatography and submitted separately to immunoprecipitation with antibodies raised against the spinach chloroplastic GS. Constant amounts of GS_1 and GS_2 activity (0.83 nkatal) were incubated with increasing volume of either antiserum or nonimmune serum. Samples were incubated for 1 h at 37°C, then overnight at 4°C, and finally centrifuged at 10,000*g* for 15 min. GS activity was assayed in the supernatant. A, GS from spinach leaves (●). B, GS_1 from rice leaves (○); GS_2 from rice leaves (●). A and B, control with nonimmune serum (■).

antibodies. This latter result is comparable to that obtained for spinach (Fig. 3A). This result suggests that GS_2 isolated from a number of different species are similar. Moreover, this immunochemical approach shows that GS_1 and GS_2 in several vascular plants are different proteins. This confirms the differences already obtained with comparative studies of their kinetic and regulatory properties (6, 13, 15).

It has been suggested that GS_1 is responsible for the reassimilation of ammonia produced during photorespiration (6, 12, 15) and also in glutamine synthesis in the dark (6). GS_2 has been suggested to function mainly in primary ammonia assimilation in the chloroplast. This activity is probably a light-dependent process controlled by ATP, Mg^{2+}, and H^+ concentrations in the stroma (6, 10). Very recently, it has been demonstrated that the chloroplast has the capacity to directly reassimilate ammonia released during photorespiration (2, 28). Furthermore, it has been demonstrated that the level of GS activity in the chloroplast is much higher than glutamate synthase (25) and could play a role in plastid ammonia detoxification in addition to its assimilatory function (5, 22). Clearly defined physiological roles of cytosolic and chloroplastic GS are more complicated than was first proposed in pea leaf cells (12, 27).

In several achlorophyllous plant parasites, only GS_1 is present Table I, (group A). This suggests that in these plants, glutamine synthesis can occur only in the cytosol which is comparable to the pattern observed in etiolated tissue where GS_2 activity is low (6, 7) or absent (8, 15). Moreover in nonphotosynthetic tissues such as roots, it has been shown that GS occurs in the cytosol (20, 26). These results suggest that GS_1 does not function exclusively in the reassimilation of ammonia released during the decarboxylation of glycine to serine but is also capable of primary ammonia assimilation. In constrast, some plants have only GS_2, the chloroplastic isoform Table II, (group B) and this obviously implies that ammonia resulting from any deamination of glycine must be transported to the chloroplast for reassimilation. This result is in agreement with those already obtained for spinach leaves (2, 28) where it has been shown that the chloroplastic GS is present in sufficient quantities to allow the reassimilation of ammonia resulting both from photorespiration and from nitrate reduction.

In a recent paper (17), it has been estimated that the flux of ammonia through the photorespiratory nitrogen cycle can be 5 to 10 times higher than for nitrate reduction. Therefore, the pattern of GS activity exhibited by many C_3 plants which contain less than 30% of total GS activity as GS_1 activity Table III, (group C) does not allow for the reassimilation of all ammonia released during photorespiration by cytosolic GS as described by Keys *et al.* (12) Consequently, in these plants which have high rates of photorespiration it seems likely that GS_2 must function in the reassimilation of the ammonia released during the decarboxylation of glycine to serine.

The group of plants which most closely approach the Keys *et al.* model (12) are the C_4 plants (Table IV, group D), where GS_1 activity is present at higher levels. However, photorespiration in C_4 plants as measured by CO_2 evolution is considered to be very low or absent (3). These plants have ribulose-1,5-bisphosphate carboxylase similar to that of C_3 plants and have the necessary C_2 enzymes for glycine formation and deamination. The present results suggest that C_4 plants may reassimilate photorespiratory ammonia via cytosolic GS. Furthermore, GS activity on a fresh weight basis is very similar between C_3 and C_4 plants. This suggests that GS_2 must play a role in the reassimilation of ammonia arising from the photorespiratory nitrogen cycle in the majority of C_3 plants examined.

In the results presented here, four groups of plants displaying different GS isoform complements could be distinguished, characterized mainly by the increasing importance of GS_1. Clarification of the possible physiological roles of GS_1 and GS_2 suggested in the present paper will require further experimentation, particularly with influence of light intensity, daylength on C_4, and tropical plant nitrogen metabolism. It also seems worthwhile to determine the differential gene expression of GS_1 and GS_2 among the four different groups of plants.

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